

THE EFFECT OF AGE AND LEAF POSITION ON CARBON DIOXIDE COMPENSATION POINT (Γ), AND POTENTIAL PHOTOSYNTHETIC CAPACITY, PHOTORESPIRATION AND NITRATE ASSIMILATION IN *HORDEUM VULGARE* L.

PATRICK FAIR, JOHN TEW AND CHRISTOPHER CRESSWELL

(*Department of Botany, University of the Witwatersrand, Johannesburg*)

ABSTRACT

The carbon dioxide compensation point (Γ) was found to vary with age under constant environmental conditions. The activities of ribulose 1-5, diphosphate carboxylase, glycollate oxidase, and nitrate reductase were found to increase with ascending leaf position. The activities were also found to fluctuate generally decreasing with age.

The use of these enzyme activities are considered as possible indicators of carbon dioxide fixation, and output during photosynthesis and the possible relationship between nitrogen metabolism and photorespiration is discussed.

UITTREKSEL

DIE UITWERKING VAN OUDERDOM EN BLAARPOSISIE OP KOOLSUUR-GASKOMPENSASIEPUNT (Γ) EN POTENTIËLE FOTOSINTESIËSE KAPASITEIT, FOTORESPIRASIE EN NITRAAT ASSIMILASIE IN *HORDEUM VULGARE* L.

Dit is gevind dat, onder konstante omgewingstoestande, die koolsuurgaskompensasiepunt met ouderdom varieer. Die aktiwiteite van ribulose difosfaat karboksilase, glikolaat oksidase en nitraat reduktase neem toe met stygende blaarposisie. Dit is ook gevind dat die aktiwiteite 'n skommeling toon, met 'n algemene afname namate die blaar ouer word.

Hierdie ensiemaktiwiteite kan moontlik as indikators van koolsuurgasfiksering en -produksie tydens fotosintese gebruik word. Die moontlike verwantskap tussen stikstofmetabolisme en fotorespirasie word bespreek.

INTRODUCTION

Recent evidence has strongly supported the concept of photorespiration occurring in green plant tissue (Ludwig and Calvin, 1971; Zelitch, 1968; Tregunna, Krotkov and Nelson, 1966; Forrester, Krotkov and Nelson, 1966). It is thought that this process is associated with single membrane microbodies termed peroxisomes (Tolbert and Yamazaki, 1969). Tolbert (1971) has recently suggested the enzymes associated with peroxisomal metabolism. Further it is generally accepted that photorespiration influences the carbon dioxide compensation point (Γ) (Jackson and Volk, 1970).

The process of photorespiration is markedly influenced by the oxygen concentration, the process being stimulated by increasing oxygen levels. Thus, the carbon dioxide compensation point (Γ) is also affected by the oxygen concentration in the environment (Forrester *et al*, *loc. cit.*). Suggested schemes for the influence of oxygen on photorespiration have been put forward by Ogren and Bowes (1971) and Plaut and Gibbs (1970).

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It has been generally accepted that the Γ of a leaf under constant environmental conditions is stable, however, the work presented in the paper suggests that this may not be the case. In an attempt to study this further, the level of carbon dioxide uptake (photosynthesis) and output (photo-respiration) was studied, by observing the enzyme activities associated with these two processes together with the Γ . The activity of the enzyme ribulose 1—5, diphosphate carboxylase (4.1.1.f.) was used to assess the level of carbon dioxide uptake, as it is accepted as the main photosynthetic carboxylating enzyme in Calvin Cycle (C3) plants. The selection of marker enzymes for the decarboxylation process was more complex. Glycollate oxidase (1.1.3.1.) is thought to be one of the main terminal oxidases associated with photorespiration (Tolbert and Yamazaki, 1969) however, the measurement of its activity need not necessarily give a measure of the level of decarboxylation due to the glyoxylate—glycollate shunt brought about by another peroxisomal enzyme NADP* glyoxylate reductase (1.1.1.26) (Tolbert and Yamazaki, *loc. cit.*) or alternatively the formation of glycine from glyoxylate and its subsequent removal from the peroxisome in this form. In the scheme suggested by Tolbert for peroxisomal metabolism, associated with photorespiration, glutamic acid plays a major role. Further as glutamic acid is considered to be the prime product of reductive amination, it was decided to investigate the possibility of using the enzyme nitrate reductase (1.6.6.2) as a measure of peroxisomal activity in plants grown with nitrate as the sole nitrogen source.

MATERIAL AND METHODS

Barley plants, *Hordeum vulgare* L. (Sunblest Seed, Desert Seed Co., El Centro, U.S.A.) were grown in soil in 10 cm diameter pots supplemented daily with Long Ashton nutrient medium (Hewitt, 1952) under constant environmental conditions of 14 hours of light at 27°C and 10 hours of dark at 22°C. During the first days the plants were thinned to leave only plants of equal height. Two growth chambers were used with a staggered light regime, so that sampling could be made twice a day, after the plants had only received 4 hours of light. Twenty four hours prior to sampling, the plants were supplied with 100 mls nitrate solution containing 500 p.p.m. nitrate.

Sampling commenced after the first leaf (Leaf 1) was longer than 12 cms. This leaf was only fully expanded four days after the initial sample. The same procedure was followed for leaves 2, 3 and 4. Leaf 4 being the uppermost leaf to appear on the axis.

Sixteen similar leaves in age and leaf position were used for each set of determinations, eight of which were used to determine the Γ , and the remainder for enzyme activity.

*Nicotinamide adenine dinucleotide phosphate.

Determination of carbon dioxide compensation point: Eight freshly sampled leaves were placed with their cut ends in a 10 ml beaker containing deionised water. The water surface was coated with wax to prevent the buffering effect of the water on the carbon dioxide. The leaves were then placed in a sealed glass chamber with a constant light source of 20 000 lux, and the temperature maintained at 30°C. The carbon dioxide compensation point was measured using a Hartmann and Braun I.R.G.A. in a closed system. Oxygen was also measured using a Hartmann and Braun paramagnetic oxygen analyser. The oxygen level remained constant at 21 per cent throughout the experiment.

Enzyme extract: 500 mg of freshly cut leaf material was ground in 10 ml of cold grinding media, using a chilled mortar and pestle with acid washed sand. The grinding medium was a modified Breidenbach, Kahn and Beevers (1968) medium containing 0,15 M Tris-HCl buffer (pH 7,5), 0,01 M EDTA (pH 7,15), 0,01 M KCl, 0,001 M $MgCl_2$, and 10 mM Dithiothreitol. The extract was squeezed through cheese-cloth, and centrifuged at 1 000 g for 5 minutes. The supernatant was used as a crude enzyme source. All the above procedures were carried out at 0°C.

Assay of ribulose 1—5, diphosphate carboxylase (4.1.1.f): A modified Bjorkman (1968) method was employed, measuring the incorporation of sodium carbonate C-14 as a measure of enzyme activity. 0,05 ml of enzyme extract was incubated at 25°C for four minutes in the presence of 20,0 μ moles $NaH^{14}CO_3$ (0,5 μ Cl: μ mole⁻¹), 0,2 μ moles ribulose 1—5, diphosphate (Sigma Chemical Co.), 30,0 μ moles Tris-HCl buffer (pH 8,0), 3,0 μ moles $MgCl_2$, and 0,1 μ mole EDTA. The reaction was carried out directly in the glass scintillation vial, in a total volume of 0,45 ml. The reaction was stopped, and the excess bi-carbonate removed by the addition of 0,2 ml of 6,0 N acetic acid. The solution was evaporated to dryness using a dry air stream for 30 minutes. 0,1 ml of deionised water was added to redissolve the residue. 15,0 ml scintillation fluid (Lips and Beevers, 1966a, 1966b) was finally added, and the number of disintegrations per minute counted on a Packard Tri-Carb Scintillation Spectrometer. A blank without enzyme, and a control in the absence of ribulose 1—5, diphosphate were carried out with each determination.

Assay of glycolate oxidase (1.1.3.1): The assay was a modification of McNaughton and Fullem (1970) method. 0,3 ml of extract was incubated at 25°C in a 0,067 M. Phosphate buffer (pH 7,0) with 10,0 μ moles sodium glycolate (B.D.H.). The total volume was 3,0 ml. The rate of oxygen uptake was measured by a Clarke Polarographic oxygen electrode. The buffer with or without glycolate was allowed to equilibrate with atmospheric oxygen for three minutes prior to being sealed by the monitoring oxygen electrode. The reaction was initiated by the injection of the enzyme extract. The reaction was followed

for five minutes, and the activity expressed as nett percentage oxygen taken up per minute, the dissolved oxygen content being taken as 100 per cent.

Assay of Nitrate Reductase: The method of Hewitt and Nicholas (1964) was employed.

Protein determination: The soluble protein was estimated colormetrically by the Folin-Ciocalteu reagent, by the method of Lowry, Rosebrough, Farr and Randall (1951).

All enzymatic activities have been expressed per gram fresh weight. The method was similar to that of Downton and Slatyer (1971). Grinding was tested for its variability against protein and the standard deviation for 20 grinds was less than 1%.

RESULTS

The effect of age on Γ , ribulose 1—5, diphosphate carboxylase, glycollate oxidase, and nitrate reductase activities for leaves 1, 2, 3 and 4, are presented in Figures 1—4.

Carbon dioxide compensation point (Γ). The effect of leaf age on Γ is shown in Graph 'b' of Figures 1—4. The compensation point was observed not to be at a constant level of carbon dioxide, but rather fluctuated with respect to time. In leaves 1, 3 and 4 the initial compensation point was at a higher carbon dioxide level, than at later stages of leaf development. A possible reason for leaf 2 not showing this high initial level was that it was more mature (± 15 cms) than the other leaves when it was first sampled. The pattern of Γ variation for the four different leaves, was found to vary and did not give a common pattern.

The average Γ for each leaf is found to be approximately the same for all the leaves and does not decrease with age. This is of particular interest when compared to the enzyme activities.

Ribulose 1—5, diphosphate carboxylase (4.1.1.f) activity: The effect of age and leaf position on the activity of this enzyme is presented in Graph 'a' Figure 5. The activity is found to increase with leaf position of the main axis, and decreases with leaf age. Activity was observed to fluctuate rhythmically on a frequency common to all leaves, but the amplitude of the fluctuation increases with leaf number.

There appears to be no correlation between the Γ and ribulose 1—5, diphosphate carboxylase activity observed.

Glycollate oxidase (1.1.3.1) activity: The influence of leaf age and position on the activity of this enzyme is presented in Graph 'c' Figure 5. Like ribulose 1—5, diphosphate carboxylase the activity is found to increase with leaf position up the main axis, and to decrease with ageing of the leaf. The activity does fluctuate with age, but does not follow the regularity observed with the carboxylating enzyme. Again no correlation could be obtained between the observed activity of this enzyme, and the Γ measured.

Nitrate reductase (1.6.6.2) activity: The effect of leaf age and position on the activity of this enzyme is presented in Graph 'b' Figure 5. Like the previous two enzymes, nitrate reductase activity is observed to increase with leaf position up the main axis, and the activity fluctuates as it ages, with no regular pattern, but with a decrease in activity as ageing occurs. Also no direct correlation between nitrate reductase activity and Γ was found.

Ratio of Glycollate oxidase activity to ribulose 1—5, diphosphate carboxylase: It was argued that if ribulose 1—5, diphosphate carboxylase activity was directly related to carbon dioxide fixation, and glycollic acid oxidase activity was a measure of photorespiration, then the ratio of these two activities should be related to the measured Γ . The ratio of the two enzyme activities are plotted for each leaf in Graph 'c' of Figures 1—4. When the plots of the ratio of these two enzyme activities are compared with the measured Γ , a weak correlation is obtained. The points which do not correlate have been indicated on Graph 'c' of Figures 1—4.

Ratio of nitrate reductase activity to ribulose 1—5, diphosphate carboxylase activity: If nitrate reductase activity is used as an indirect indicator of photorespiration using the reasoning given in the introduction, and the ratio of this enzyme activity to ribulose 1—5, diphosphate carboxylase activity is plotted, the results are presented in Graph 'a' Figures 1—4. When the ratio of these two enzymes activity is compared with the observed Γ , a reasonably good correlation was obtained.

The percentage correlation of the calculated ratios of Glycollate oxidase activity/ribulose 1—5, diphosphate carboxylase activity to Γ , and nitrate reductase activity/ribulose 1—5, diphosphate carboxylase activity to Γ , are presented in Table 1. These results show the improved correlation obtained when nitrate reductase activity was used as an indirect indication of photorespiration.

DISCUSSION

The results indicate that the carbon dioxide compensation point Γ , under constant environmental conditions varies with the age of the leaf and the position of the leaf on the main axis. This variation in Γ suggests that the equilibrium of carbon dioxide uptake and output is probably in a continual state of flux.

The fluctuation of the Γ cannot be explained by the observed ribulose 1—5, diphosphate carboxylase activity in Graph 'a' Figure 5. The activity of this enzyme shows a rhythmic fluctuation, which is not observed in the measured Γ . The rhythmic pattern observed for the carboxylating enzyme, which had a frequency common to all the leaves investigated, may correspond to periods of high and low demand for carbon skeletons, respectively during the growth of the plant. In addition, the pattern of ribulose 1—5, diphosphate carboxylase

TABLE 1

The percentage positive correlation of the with the calculated ratios of the four leaves sampled.
 (a) The %-age correlation of the glycollate oxidase: ribulose 1-5, diphosphate carboxylase ratio

(i) on a relative basis

(ii) on a nonproportional basis (i.e. when the direction of change and not the magnitude is considered)

(b) The %-age correlation of the nitrate reductase: ribulose 1-5, diphosphate carboxylase ratio (i) and (ii) as for a.

LEAF NO.	% CORRELATION WITH Γ			
	a (i)	b (i)	a (ii)	b (ii)
1	27	47	53	73
2	54	69	77	85
3	64	75	83	91
4	22	79	67	89

activity and Γ do not correspond, with respect to leaf position. The carboxylating enzyme activity was found to increase with each new leaf formed, Graph 'a' Figure 5, whereas the average Γ was observed to remain relatively constant irrespective of leaf position. Under these conditions, if photorespiration remained constant, one would have expected the Γ to decrease, with increased carboxylating activity found with each new leaf produced, but this was not observed to be the case. This observation, together with observed rhythmic fluctuation of carboxylating enzyme activity, further supports the idea that the equilibrium of carbon dioxide uptake and output is probably in a continual state of flux.

Both glycollate oxidase activity and nitrate reductase activity alone, show no correlation with the observed Γ . Unlike the carboxylating enzyme, the activity of these two enzymes show no rhythmic pattern, and the pattern of activity varies from leaf to leaf. However, the overall levels of activity of these enzymes also rise with each new leaf formed of those studied, and exhibit a general decrease in activity with age, indicating a possible relationship with ribulose 1-5, diphosphate carboxylase activity. The observed rise in glycollate

FIG. 1.

Leaf 1. The effect of age on the Γ (Graph 'b'), and the enzymes nitrate reductase, glycollate oxidase and ribulose 1-5, diphosphate carboxylase (Graph 'd'). Graph 'a' is the calculated ratio of nitrate reductase to ribulose 1-5, diphosphate carboxylase (●—●). Where this ratio is divergent from the Γ the expected position is marked by a clear circle (○ ... ○) and numbered (top row). The lower row of numbers represents those points which are divergent when correlated on a nonproportional basis (i.e. when the direction of change and not the magnitude is considered). These points are marked by a dashed line and clear triangle (△—△) where necessary. Graph 'c' is the calculated ratio of glycollate oxidase to ribulose 1-5, diphosphate carboxylase (●—●). The explanation of the graph is as for graph 'a'.

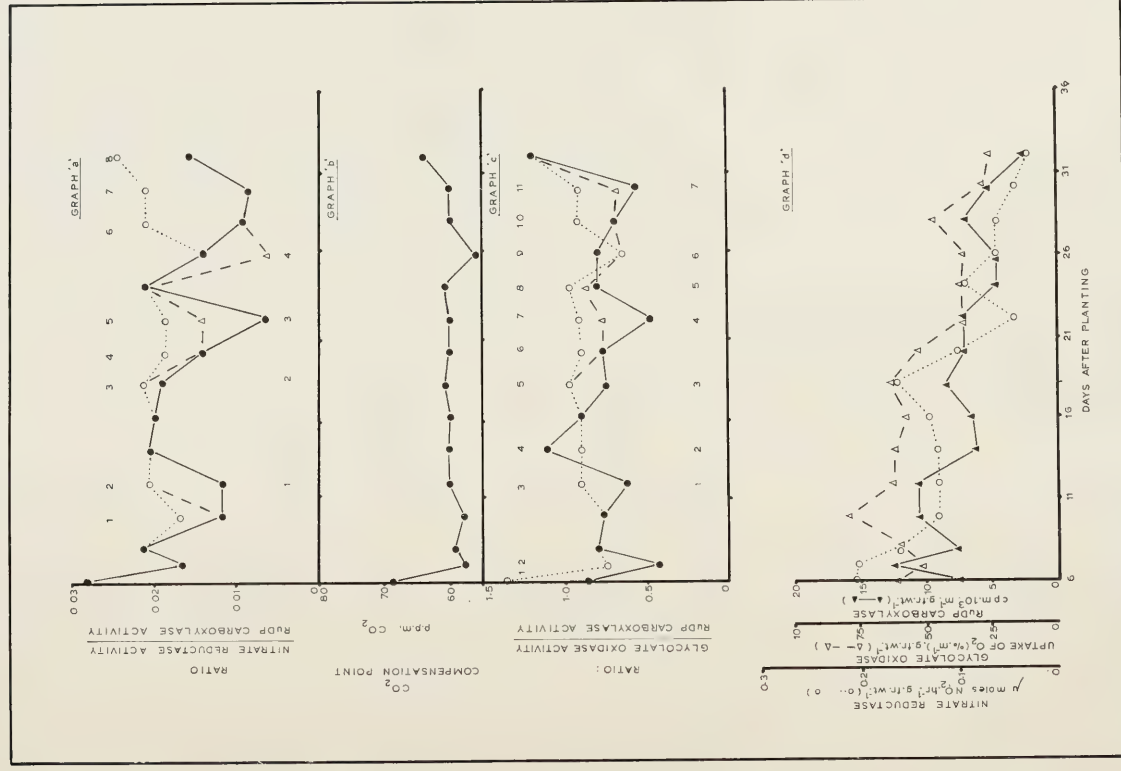


Fig. 1.

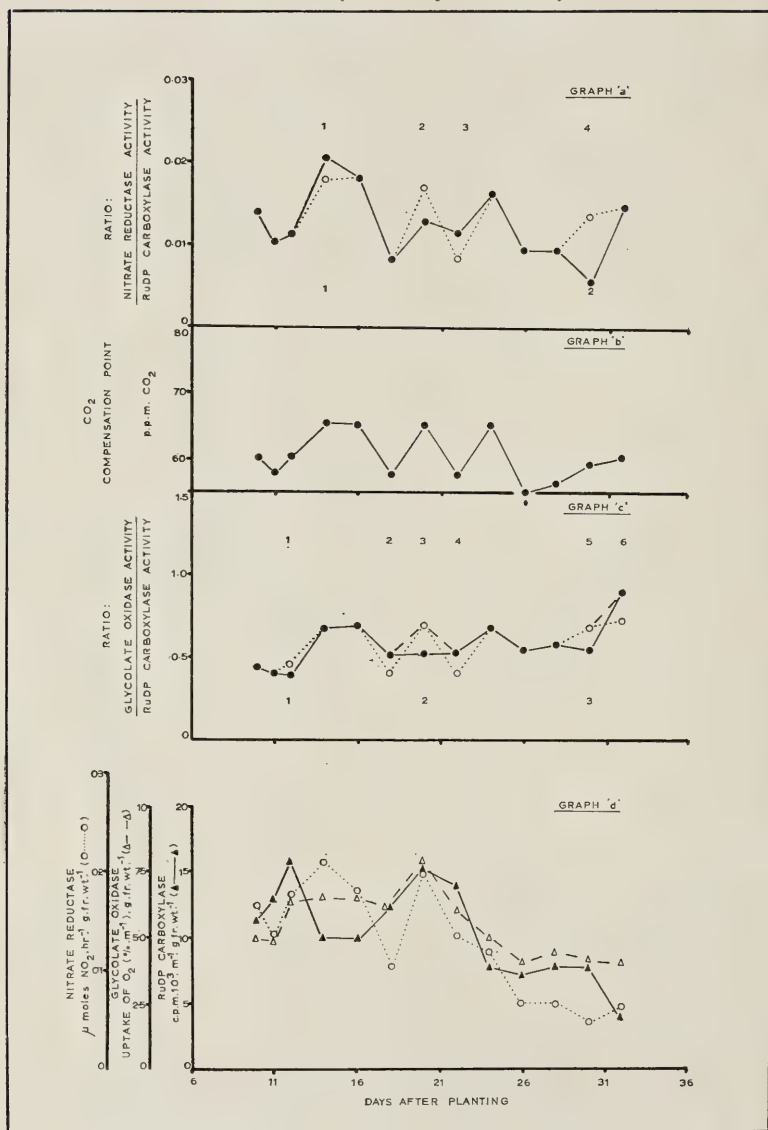


FIG. 2.
Leaf 2. Explanation as for Fig. 1.

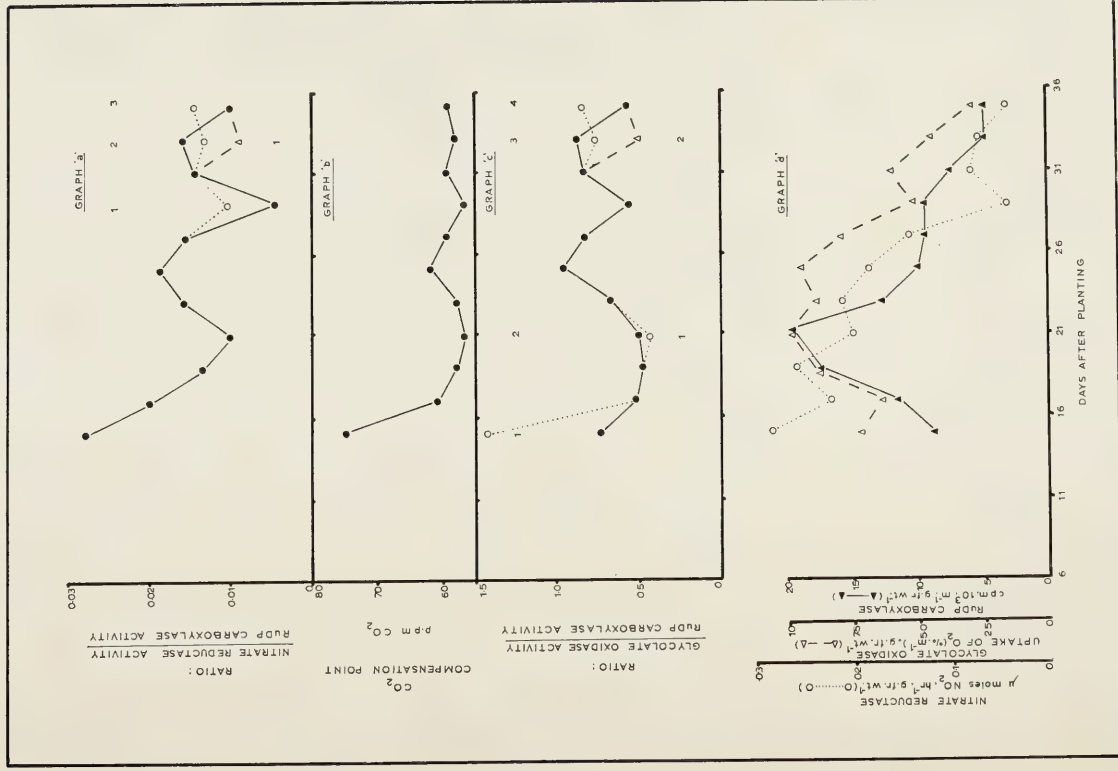


Fig. 3.
Explanation as for Fig. 1.

oxidase, and nitrate reductase did not result in any general rise in Γ . The observed increase in nitrate reductase activity with ascending leaf position, differs from the observations made by Carr and Pate (1967) using 'field pea'. As the enzymes ribulose 1—5, diphosphate carboxylase is directly responsible for carbon dioxide fixation, and glycollate oxidase has been suggested as being directly involved in photorespiration, it was anticipated that the ratio of these two enzymes would give a good correlation with the observed Γ . However, when the ratio of these two enzyme activities was determined, Graph 'c' Figures 1—4, only a low order of correlation was observed, particularly in leaves one and four. A possible explanation for this observation, is that the enzyme glycollate oxidase participates in the glycollate—glyoxylate shunt as well as in the decarboxylation pathway involved in photorespiration, as suggested in the proposed photorespiration pathway (Tolbert *et al*, 1969).

However, when the ratio of nitrate reductase activity to ribulose 1—5, diphosphate carboxylase activity was plotted Graph 'a' Figures 1—4, and this compared with the observed Γ , a greatly improved correlation was found. This suggests that nitrate reductase activity is indirectly related to photorespiration activity in plants receiving their nitrogen solely as nitrate. Further, this result suggests that under these conditions nitrate reductase activity, is more reliable than glycollate oxidase activity as an indirect measure of photorespiration, due to the product of nitrate assimilation being probably associated with amination steps thought to be involved in photorespiration. If this is the case, then it is thought a major function of photorespiration could be the supply of carbon skeletons from glycollate for amino acid metabolism in the light. Under these circumstances a relatively high Γ as observed in the early stages of developing leaves, could indicate carbon flow into amino acid formation, rather than sugar formation, when a lower Γ would be expected.

Further indirect support for a possible connection between nitrate assimilation and peroxisomal metabolism is suggested by Ruis and Kindl (1971) who suggest from their results that leaf peroxisomes may have more importance in amino acid metabolism than was previously assumed. Beevers and Hagerman (1969) observed that oxygen was essential for the assimilation of nitrite, this could be explained by the fact that oxygen is required for photorespiration, which we suggest is associated with nitrite assimilation. In labelling experiments using $C^{14}O_2$ Ongun and Stocking (1965) observed that after fifteen minutes of photosynthesis of the amino acids present in the leaf, the labeled carbon was predominantly in the soluble amino acid fraction namely serine, glycine, aspartate and alanine. All these amino acids are involved in the proposed photorespiration pathway, Figure 6.

Based on the above we suggest that the level of Γ could therefore be con-

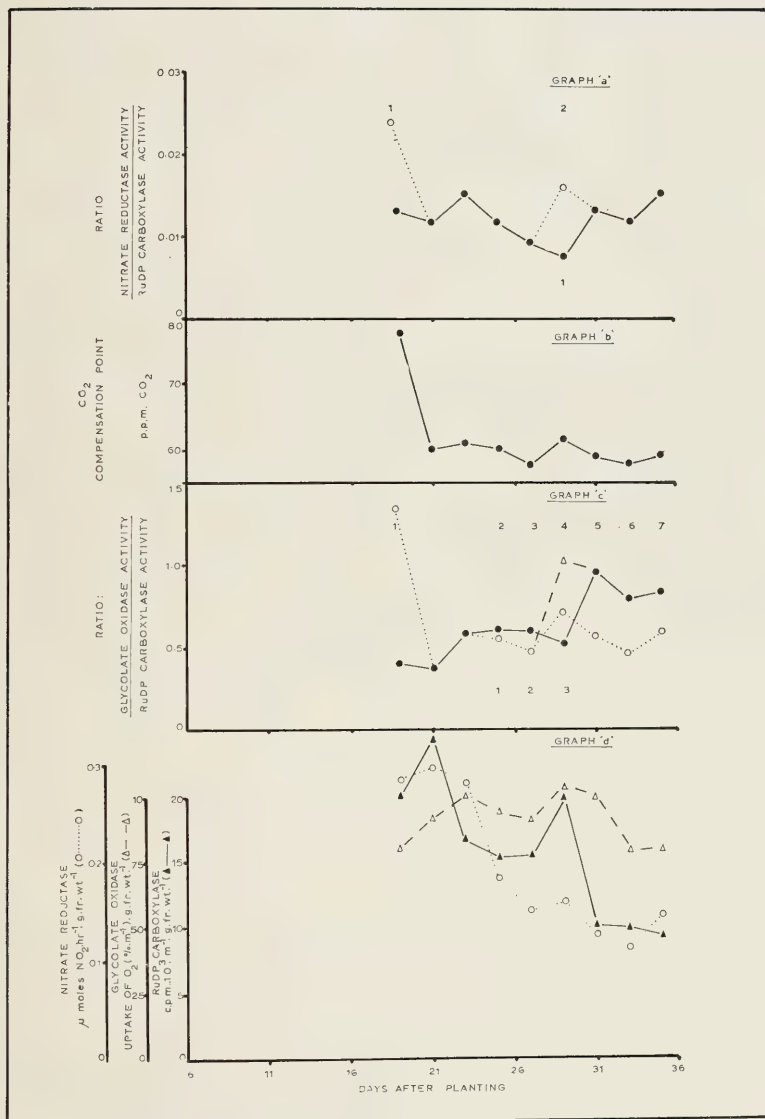


FIG. 4.
Leaf 4. Explanation as for Fig. 1.

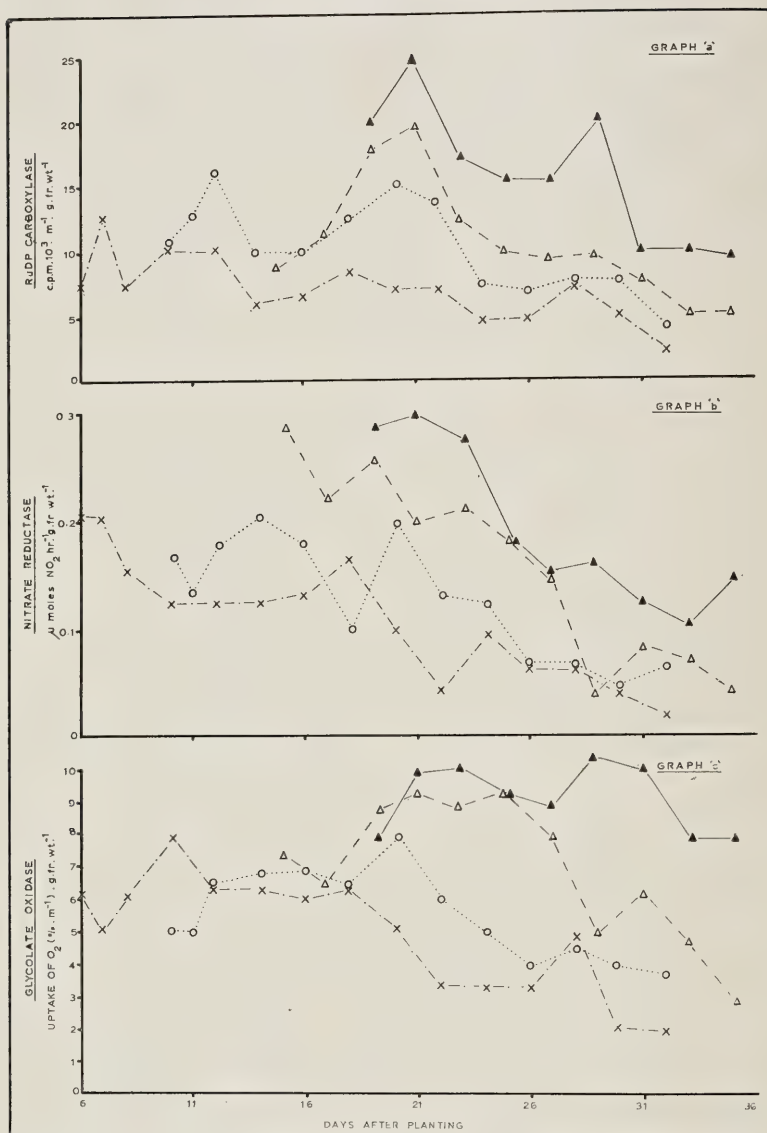


FIG. 5.

The effect of age on the ribulose 1-5, diphosphate carboxylase (Graph 'a'), nitrate reductase activity (Graph 'b') and glycolate oxidase activity (Graph 'c') for the four leaves; sampled. Leaf 1 (x-x-x); Leaf 2 (○...○); Leaf 3 (△-△); Leaf 4 (▲-▲).

trolled by the demand for either amino acids or sugars. It could also be regulated by the level of available nitrogen.

Finally, it is suggested that the significance of nitrate reductase activity as a possible indirect measure of photorespiration could be affected in one or all of the following ways:

- (a) The product of nitrate assimilation may be utilized outside the peroxisome.
- (b) The influence of nitrogen supply for amino acid formation from sources other than the peroxisomal cell nitrate assimilation.

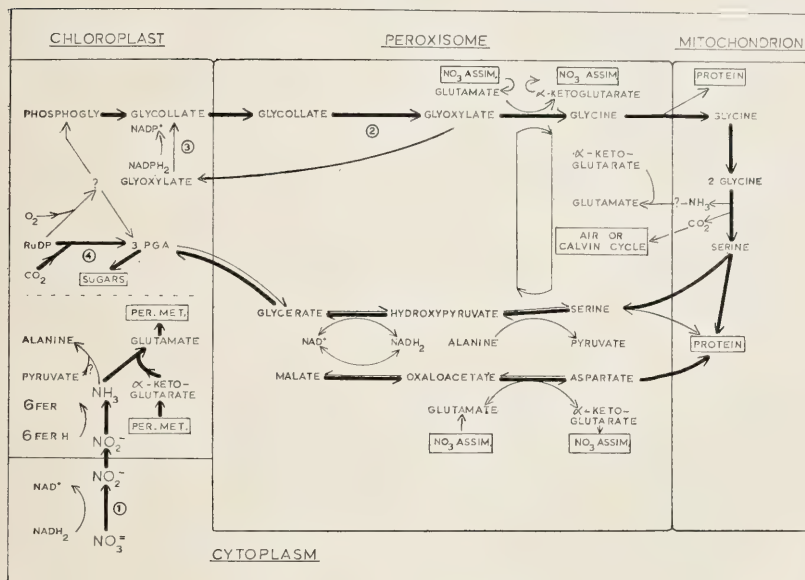


FIG. 6.

Proposed flow diagram modified from Tolbert (1971). (1) Nitrate reductase; (2) Glycollate oxidase; (3) NADPH-glyoxylate reductase; (4) ribulose 1-5, diphosphate carboxylase; NO₃ ASSIM-Nitrate assimilation; Per. Met. -Peroxisomal metabolism.

(c) The formation of serine may occur through the reverse reaction sequence namely 3 Phosphoglyceric acid → Glyceric acid → Hydroxypyruvate → Serine as shown in Figure 6.

(d) Glycine may be utilized directly prior to the decarboxylating step as shown in Figure 6.

CONCLUSIONS

The results of the work presented, demonstrate the following points:

- (1) CO₂ compensation point is not a fixed point, but fluctuates.
- (2) Ribulose 1—5, diphosphate carboxylase activity varies as follows:
 - (i) Activity increases with each successive leaf studied.
 - (ii) Activity shows a rhythmic pattern of fluctuation, synchronized in the four leaves studied.
 - (iii) A general decline in activity occurs as a leaf ages.
- (3) Overall levels of glycollate oxidase and nitrate reductase activity increase within each succeeding new leaf studied up to leaf four.
- (4) The overall level of glycollate oxidase and nitrate reductase activity drop steadily as a leaf ages.
- (5) Nitrate reductase activity appears to be closely coupled with photorespiration in barley plants supplied with nitrate as sole source of nitrogen.

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